Rapid, High-Throughput Detection of Azalea Lace Bug (Hemiptera: Tingidae) Predation by *Chrysoperla rufilabris* (Neuroptera: Chrysopidae), Using Fluorescent-Polymerase Chain Reaction Primers

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ABSTRACT Azalea lace bugs, *Stephanitis pyrioides* (Scott) (Hemiptera: Tingidae), are the most common pest of azaleas (*Rhododendron* spp.) in nursery production and the landscape. Although pesticides are commonly used to control lace bugs, natural enemies can be a significant source of lace bug mortality. Lacewings (Neuroptera: Chrysopidae) are natural enemies of lace bugs and easily consume them in laboratory studies. Field studies on lacewing biocontrol of azalea lace bugs are underway; however, monitoring lacewing predation in a nursery environment by direct observation is impractical. Here, we describe a fluorescent-polymerase chain reaction method to estimate *S. pyrioides* consumption based on the gut contents of lacewing predators. Lace bug DNA was detected in fed lacewings up to 32 h after ingestion. More than 80% of the ingested lace bugs were detected using our method with only one false positive result. The assay is both high-throughput and relatively inexpensive, making it a practical approach to documenting lace bug predation in the field.

KEY WORDS biological control, molecular detection, fluorescence, azaleas, *Stephanitis pyrioides*

The azalea lace bug, *Stephanitis pyrioides* (Scott) (Hemiptera: Tingidae), is the most important pest of azaleas in landscapes and nurseries (Neal and Douglass 1988). Adults and nymphs feed by inserting their stylet bundle through stomata on the abaxial side of leaves, and they remove cell contents from the parenchymal layer. The damage is evident as chlorotic stippling on the adaxial side of the leaves (Klingeman et al. 2000).

Current control measures for landscapes and nurseries emphasize pesticide applications, especially early in the season (Neal and Schaefer 2000, Shrewsbury and Smith-Fiola 2000). Mistimed applications of pesticides, especially broad-spectrum pesticides, reduce populations of beneficial arthropods, which can lead to rapid resurgence of the targeted pest and occurrence of secondary pests (Metcalf 1994).

Most lace bugs have few specific natural enemies (Neal and Schaefer 2000). Specific natural enemies of the azalea lace bug, however, include the wasp *Anagrus takeyanus* Gordh (Hymenoptera: Mymaridae) (Balsdon et al. 1996) and the predacious mirid *Stethoconus japonicus* Schumacher (Hemiptera: Miridae) (Henry et al. 1986). Generalist predators known to feed on azalea lace bug include *Rhinocapsus vanduzeei* Uhler (Hemiptera: Miridae) (Stewart et al. 2002) and the lacewings *Chrysoperla rufilabris* Burmeister and *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) (Shrewsbury and Smith-Fiola 2000, Stewart et al. 2002). Many other generalist predators, especially spiders (D.W.B., personal observation), are found on azaleas that could possibly feed on azalea lace bugs. For example, Klingeman et al. (2001) listed 64 potential azalea lace bug predators collected from azaleas in just one county of Georgia, U.S.A.

Natural enemies of lace bugs could be used for biocontrol, either by introducing them into the environment or by encouraging growth of their populations. Molecular techniques have been developed that successfully detect species-specific prey in the guts of insect predators (for review, see Symondson 2002). Examples include monoclonal antibodies to detect aphids (Symondson et al. 1999) and genomic or mitochondrial gene polymerase chain reaction (PCR) primers to detect prey in guts of predators (Zaidi et al. 1999, Chen et al. 2000). Although indirect, molecular techniques based on gut content analysis can be used on a large scale to estimate predation in real world environments.

We developed a relatively inexpensive, high-throughput method to detect lace bug DNA in the gut contents of predators. We choose to use a PCR-based technique because it could be rapidly developed and

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made specific to the azalea lace bug. Hundreds of copies of mitochondrial genes are present in each cell because of the variable number of mitochondria, as opposed to nuclear genes, which are restricted to two copies in a typical diploid cell. To that end, we designed fluorescent-labeled primers specific to a portion of the mitochondrial cytochrome oxidase I gene of azalea lace bug and verified their usefulness, especially in lacewing larvae.

Materials and Methods

C. rufilabris larvae were obtained by overnight delivery from an insect supplier (Beneficial Insectary, Oak Run, CA). Third instars were shipped in rearing frames (corrugated cardboard), with each larva in a single cell, and with eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) provided as food. *S. pyrioides* were collected from various cultivars of azaleas in Pearl River Co., Poplarville, MS. Voucher specimens of lace bugs and lacewings were placed in the National Museum of Natural History, Washington D.C. *Corythuca ciliate* (Say) (Hemiptera: Tingidae) was collected from Pearl River Co., MS, and *Teleonemia scrupulosa* Stål (Hemiptera: Tingidae) was collected from Mobile Co., AL, and George and Pearl River counties, MS.

Third instars of *C. rufilabris* were placed in 50- by 9-mm tight-fit lid petri dish (Falcon; BD Biosciences Discovery Labware, Bedford, MA) and starved overnight (to increase appetite) by placing them in an incubator (I-30BLL, Percival Scientific, Inc., Perry, IA) set at 27°C, 60% RH, and a photoperiod of 16:8 (L:D) h. One *S. pyrioides* adult was added the next day. The time each lacewing began feeding on the lace bug and the time it stopped feeding were recorded. The lacewings were placed in 0.5 ml of 100% ethanol (E702-3. Aldrich, Milwaukee, WI) at time intervals of 0, 2, 4, 6, 8, 16, 24, and 32 h. Ten replicates were attempted for each time, and the experiment was repeated once. Other predators, often collected with azalea lace bugs (D.W.B., unpublished data), were tested after a few minutes of feeding on an azalea lace bug. These predators included a lynx spider, *Peucetia* sp. (Arachnida: Araneae: Oxyopidae); a ladybeetle adult, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae); and two assassin bugs, *Sinea diadema* (F.) and *Zelus longipes* (L.) (Hemiptera: Reduviidae).

Ethanol-preserved insects were dried for 5 min in a DNA110 Speedvac to remove residual alcohol (Thermo Electron Corporation, Waltham, MA). Tissue was pulverized by shaking at a frequency of 30 cycles per second for 5 min in a Retsch Mixer Mill 301 (Retsch Inc., Haan, Germany) using 2-ml screwcap tubes with a 5-mm tungsten bead (QIAGEN, Valencia, CA). Total genomic DNA was extracted using UltraClean Soil DNA extraction kit with PCR inhibitor as per manufacturer protocol (Mo Bio Laboratories, Carlsbad, CA). DNA was quantified using PicoGreen dye (Invitrogen, Carlsbad, CA) and a Turner Quantech digital fluorometer (Barnstead International, Dubuque, IA).

The cytochrome oxidase I gene (COI) was amplified using primers C1-N-2096 and C1-J-1763 (Kambhampati and Smith 1995, Lunt et al. 1996, Zhang and Hewitt 1997, Bonacum et al. 2001). Amplified fragments were purified using QiaQuick columns (QIAGEN) and sequenced directly using BigDye version 3.1 (Applied Biosystems, Foster City, CA). Data from both strands were aligned and verified accurate using Sequencher (Gene Codes, Ann Arbor, MI). Edited sequences were deposited in GenBank under accession numbers DQ363338 through DQ363373.

We aligned 321 bp of DNA sequence data from 36 samples, including 10 *S. pyrioides*, eight *C. rufilabri*, nine *C. ciliate*, and nine *T. scrupulosa*, by using MegAlign (DNAstar, Madison, WI) using ClustalW and then modified by eye. Phylogenetic reconstruction was done using maximum parsimony methods with PAUP*, version 4.05b10 (Swofford 1998). Computational parameters included heuristic search using random sequence addition and tree-bisection-reconnection (TBR) as the branch-swapping algorithm. We used 100 bootstrap replicates to generate statistical support.

Twenty-eight lace bug-specific primer combinations were designed using PrimerSelect (DNAstar). Primer combinations were tested using four different Taq polymerases and eight cycling conditions to optimize results for efficiency, cost, and reproducibility. The best results were found for LaceBugCOI-9 5′-TTGTCGTTTGGCTACTAT-3′ and LaceBugCOI-1 5′-GATTATCCTGATTTATTCT-3′ primers. The LaceBugCOI-9 primer was fluorescently labeled with FAM-5 (Integrated DNA Technologies, Coralville, IA). Genomic DNA was PCR amplified using Eppendorf MasterMix (Brinkmann Instruments, Westbury, NY). Cycling conditions using a DNA engine Tetrad Thermocycler (MJ Research, South San Francisco, CA) include 95°C for 2 min, 43 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, followed by 10 min at 72°C, and storage at 4°C. One microliter of genomic DNA was used for all amplifications; quantities ranged from 3.1 to 9.9 ng/μl. Amplified fragments were visualized on an ABI 3100-Avant (Applied Biosystems) by using ROX-500 size standard. Fragment sizing and detection was automated with GeneMapper version 3.5 (Applied Biosystems). All results were visually confirmed. The detection limit was set at 100 relative fluorescent units (rfu). Visible peaks below 100 rfu were not scored as positive for lace bug DNA.

Results and Discussion

Phylogenetic reconstruction based on 123 parsimony-informative characters confirms that species identification can be verified by COI gene data (Fig. 1). Several populations of lace bugs were sampled, including *S. pyrioides*, *C. ciliate*, and *T. scrupulosa*. *C. rufilabris* was included as an outgroup to make sure the PCR assay would not cross-react with the predator. Classification of samples from different geographic regions or populations was not possible due to

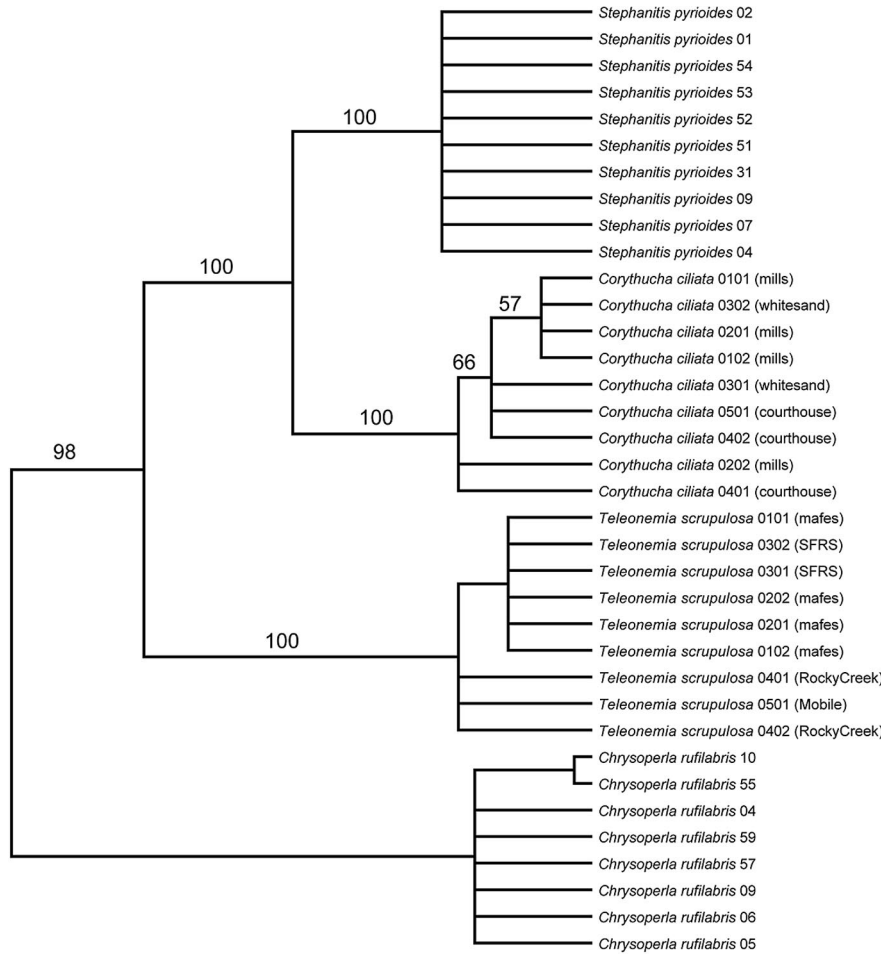

Fig. 1. Strict consensus of the 33 most parsimonious trees for lace bug and lacewing populations were created using a 321-bp portion of the mitochondrial COI gene. Bootstrap values from 100 replicates are shown above branches.

a lack of variation. However, increased variation was recorded for *C. ciliate* compared with the other species. Sequence data indicated the COI gene was a good candidate for assay development because the variation observed was species but not population specific.

Azalea lace bug-specific primers were designed and tested for several different regions of the COI gene. The most consistent results were produced by variation found between the TL2-N-3014 and C1-J-2753 primers corresponding to bases 2753–3014 (Kambhampati and Smith 1995, Lunt et al. 1996). Primers that annealed to regions containing mutations unique to *S. pyrioides* were tested against all species samples shown in Fig. 2 and *Stephanitis rhododendri* Horvath and *Stephanitis takeyai* Drake & Maa to confirm *S. pyrioides* specificity. Cross-reactivity was not detected for any species, including the predator *C. rufilabris.* Of 18 different *S. pyrioides* DNAs tested, 100% amplified the correct 116-bp fragment under assay conditions.

Serial dilutions of *S. pyrioides* DNA were mixed with a 20 ng of *C. rufilabris* DNA as a noncompetitive inhibitor to simulate gut content analysis conditions. *C. rufilabris* insects were reared in the laboratory and only fed on moth eggs. Consistent lace bug DNA detection was possible down to 1 pg of lace bug DNA with a 2,000-fold excess of predator DNA in the reaction (Fig. 3). Replicate reactions using the same DNAs and condition produced identical results even for failed reactions. Two-tenths and 0.1 pg of DNA produced 65 and 20% positive results, but results were not consistent when replicated (data not shown).

Controlled feeding trials were conducted in replicates, and total DNA was subjected to PCR assay. Eight time intervals were assayed and detection was possible 32 h after ingestion (Fig. 4). One microliter of total DNA was used for each reaction regardless of DNA concentration. Lacewings that pupated within the time interval after ingestion were not assayed. All other predators that had been fed lace bugs showed positive for azalea lace bug DNA.

High-throughput, inexpensive, and rapid detection of lace bug predation in the field is useful to verifying predation events of generalist predators. To that end, we developed a PCR-based assay specific to *S. pyri-*

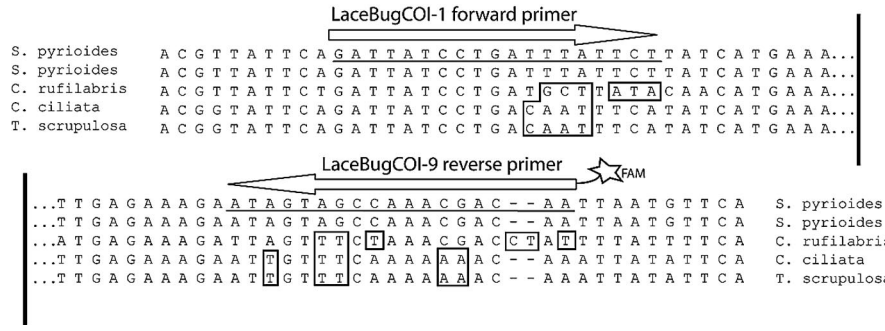

Fig. 2. Alignment of the COI gene regions where the lace bug-specific primers anneal. Nucleotides differing from *S. pyrioides* sequence data are shown in boxes. Primer direction is indicated by arrows. Total length of the PCR product is 166 bp (sequence data not shown indicated by ". . . ").

oides. PCR primers are fluorescently labeled allowing for automated detection using standard fragment analysis equipment and software. Using GeneMapper software, we manually set the signal strength cutoff to 100 rfu to eliminate false positives from low-intensity peaks or signal from other capillaries and set the analysis window to 4 bp (114–118) to reduce false positives from misprimed PCR products. Using these parameters, visual inspection of the data did not result in any changes, suggesting fully automated plus/minus detection of lace bug DNA is possible.

Assay conditions were set to reduce cost and increase sample processing. The most expensive and labor-intensive component of the assay is DNA extraction where predators must be sorted into individual vials. In this work, we used 2-ml screwcap vials, but the commercial kit and bead beating technique can be easily increased to 96-well plates for increased throughput. We optimized the assay for a premixed Taq polymerase and a broad range of input DNA. No sample cleanup is necessary before loading PCR product on the capillary sequencer instrument. The conservative cost estimate for this assay, using manufacturer-recommended methods, is $0.96 per insect. This price includes plate-based DNA extraction, fluorescent PCR amplification, capillary electrophoresis, and data analysis.

Aside from two significant differences, this research is similar to other PCR-based approaches to gut content analysis (Jarman et al. 2002, Jarman et al. 2004, Persad et al. 2004). First, a high-throughput approach using automated equipment constrains our results to simple plus/minus data. This is due in part to the optimization procedures which eliminate the need to quantify predator DNA and the detection equipment, which does not produce quantitative results. Although we can measure signal strength in rfu for each reaction, this number is not directly related to the amount of lace bug DNA in the sample. The binary nature of our assay does not detract from its usefulness in the

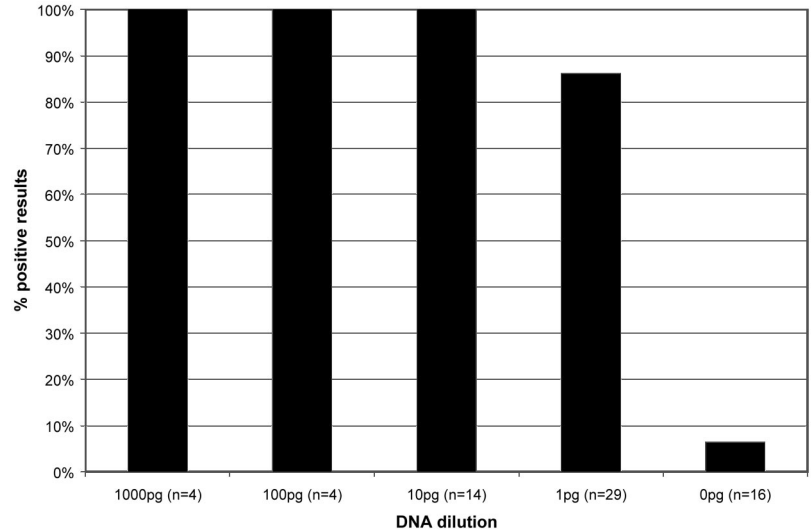

Fig. 3. Percentage of positive PCR results of serial dilutions of *S. pyrioides* DNA. All reactions included an excess of 20 ng of *C. rufilabris* DNA to simulate gut content assay conditions. One negative control produced positive results. Total number of reactions is indicated by "n = ".

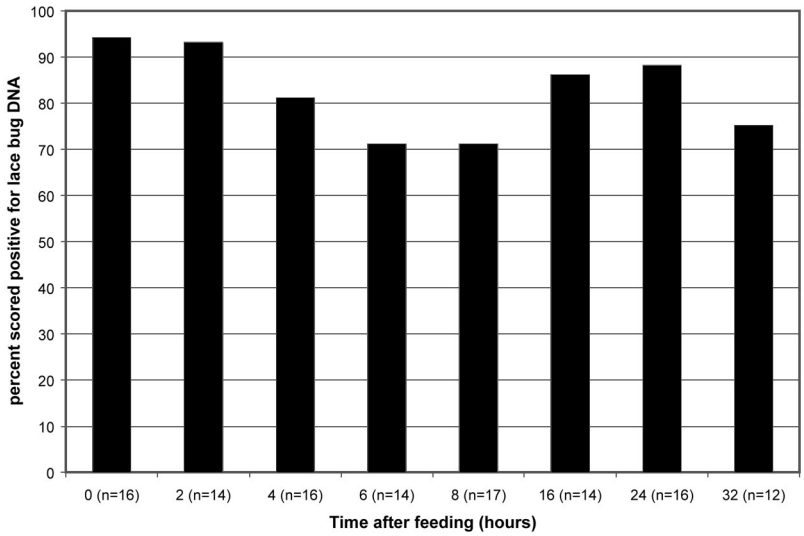

Fig. 4. Percentage of positive PCR results of *C. rufilabris* insects that fed on *S. pyrioides*. Time after feeding is indicated on the x-axis. Total number of reactions is indicated by "n = ".

field. Our assay might underestimate predation if the predator was known to consume multiple, variable numbers of lace bugs. However, collection of many predators in the field and preserving them in ethanol for future laboratory analysis is less time-consuming than watching multiple predators for countless hours, in the field. The former can provide information on how many predators fed on a particular prey though not how many prey a particular predator killed.

Second, we did not achieve 100% detection in fed lacewing samples. Surprisingly, these results are consistent when replicated. When retested, 93% of the failed reactions and 91% of the positive reactions generated identical data. Perhaps in these failed reactions, the lacewing fully digested the lace bug DNA, regurgitated the lace bug meal, or, even though it was observed to insert its mandibles, it did not actually consume any lace bug prey during feeding.

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References Cited

Balsdon, J. A., S. K. Braman, and K. E. Espelie. 1996. Biology and ecology of *Anagrus takeyanus* (Hymenoptera: Mymaridae), an egg parasitoid of the azalea lace bug (Heteroptera: Tingidae). Environ. Entomol. 25: 383–389.

Bonacum, J., R. Desalle, P. O'Grady, D. Olivera, J. Wintermute, and M. Zilversmit. 2001. New nuclear and mitochondrial primers for systematics and comparative genomics in Drosophilidae. Dros. Inf. Serv. 84: 201–204.

Chen, Y., K. L. Giles, M. E. Payton, and M. H. Greenstone. 2000. Identifying key cereal aphid predators by molecular gut analysis. Mol. Ecol. 9: 1887–1898.

Henry, T. J., J. W. Neal, and K. M. Gott. 1986. *Stethoconus japonicus* (Heteroptera, Miridae): a predator of *Stephanitis* lace bugs newly discovered in the United States, promising in the biocontrol of azalea lace bug (Heteroptera, Tingidae). Proc. Entomol. Soc. Wash. 88: 722–730.

Jarman, S. N., B. E. Deagle, N. J. Gales, and B. E. Deagle. 2004. Group-specific polymerase chain reaction for DNA-based analysis of species diversity and identity in dietary samples. Mol. Ecol. 13: 1313–1322.

Jarman, S. N., N. J. Gales, M. Tierney, S. N. Jarman, N. G. Elliott, and P. C. Gill. 2002. A DNA-based method for identification of krill species and its application to analysing the diet of marine vertebrate predators. Mol. Ecol. 11: 2679–2690.

Kambhampati, S., and P. T. Smith. 1995. PCR primers for the amplification of four insect mitochondrial gene fragments. Insect Mol. Biol. 4: 233–236.

Klingeman, W. E., S. K. Braman, and G. D. Buntin. 2000. Feeding injury of the azalea lace bug (Heteroptera: Tingidae). J. Entomol. Sci. 35: 213–219.

Klingeman, W. E., S. K. Braman, and G. D. Buntin. 2001. Azalea growth in response to azalea lace bug (Heteroptera: Tingidae) feeding. J. Econ. Entom. 94: 129–137.

Lunt, D. H., D.-X. Zhang, J. M. Szymura, and G. M. Hewitt. 1996. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. Insect Mol. Biol. 5: 153–165.

Metcalf, R. L. 1994. Insecticides in pest management, pp. 245–314. *In* R. L. Metcalf and W. H. Luckman [eds.], Introduction to pest management. Wiley, New York.

Neal, J. W., and L. W. Douglass. 1988. Development, oviposition rate, longevity, and voltinism of *Stephanitis pyrioides* (Heteroptera, Tingidae), an adventive pest of azalea, at 3 temperatures. Environ. Entomol. 17: 827–831.

Neal, J. W., and C. W. Schaefer. 2000. Lace bugs (Tingidae), pp. 85–137. *In* C. W. Schaefer and A. R. Panizza [eds.], Heteroptera of economic importance. CRC, Boca Raton, FL.

Parsed, A. B., A. Jeyaprakash, and M. A. Hoy. 2004. High-fidelity PCR assay discriminates between immature *Lipolexis oregmae* and *Lysiphlebus testaceipes* (Hymenop-

tera: Aphidiidae) within their aphid hosts. Fla. Entomol. 87: 18–24.

Shrewsbury, P. M., and D. C. Smith-Fiola. 2000. Evaluation of green lacewings for suppressing azalea lace bug populations in nurseries. J. Environ. Hortic. 18: 207–211.

Stewart, C. D., S. K. Braman, and A. F. Pendley. 2002. Functional response of the azalea plant bug (Heteroptera: Miridae) and a green lacewing *Chrysoperla rufilabris* (Neuroptera: Chrysopidae), two predators of the azalea lace bug (Heteroptera: Tingidae). Environ. Entomol. 31: 1184–1190.

Swofford, D. L. 1998. PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4. Sinauer Associates, Inc., Sunderland, MA.

Symondson, W.O.C. 2002. Molecular identification of prey in predator diets. Mol. Ecol. 11: 627–641.

Symondson, W.O.C., M. L. Erickson, J. E. Liddell, and K.G.I. Jayawardena. 1999. Amplified detection, using a monoclonal antibody, of an aphid-specific epitope exposed during digestion in the gut of a predator. Insect Biochem. Mol. Biol. 29: 873–882.

Zaidi, R. H., Z. Jaal, N. J. Hawkes, J. Hemingway, and W.O.C. Symondson. 1999. Can multiple-copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? Mol. Ecol. 8: 2081–2087.

Zhang, D.-X., and G. M. Hewitt. 1997. Assessment of the universality and utility of a set of conserved mitochondrial COI primers in insects. Insect Mol. Biol. 6: 143–150.

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